

Hetero-oligomerization between GABA_A and GABA_B Receptors Regulates GABA_B Receptor Trafficking*

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The neurotransmitter γ -aminobutyric acid (GABA) mediates inhibitory signaling in the brain via stimulation of both GABA_A receptors (GABA_AR), which are chloride-permeant ion channels, and GABA_B receptors (GABA_BR), which signal through coupling to G proteins. Here we report physical interactions between these two different classes of GABA receptor. Association of the GABA_B receptor 1 (GABA_BR1) with the GABA_A receptor γ 2S subunit robustly promotes cell surface expression of GABA_BR1 in the absence of GABA_BR2, a closely related GABA_B receptor that is usually required for efficient trafficking of GABA_BR1 to the cell surface. The GABA_BR1/ γ 2S complex is not detectably functional when expressed alone, as assessed in both ERK activation assays and physiological analyses in oocytes. However, the γ 2S subunit associates not only with GABA_BR1 alone but also with the functional GABA_BR1/GABA_BR2 heterodimer to markedly enhance GABA_B receptor internalization in response to agonist stimulation. These findings reveal that the GABA_BR1/ γ 2S interaction results in the regulation of multiple aspects of GABA_B receptor trafficking, allowing for cross-talk between these two distinct classes of GABA receptor.

GABA,¹ the primary inhibitory neurotransmitter in the mammalian brain, produces its physiological effects by acting on three different receptor subtypes: GABA_A, GABA_B, and GABA_C (1). The ionotropic receptors GABA_A and GABA_C produce fast inhibitory synaptic transmission via an intrinsic chloride channel. GABA_A receptors are pentamers composed of combinations of various subunits, with the most prevalent com-

bination in the mammalian brain containing two α 1 subunits, two β 2 subunits, and one γ 2 subunit (2). GABA_B receptors, in contrast, are metabotropic G protein-coupled receptors (GPCRs) that mediate the slow inhibitory neurotransmission of GABA via the regulation of several effectors. GABA_B receptors are believed to be heterodimeric combinations of two GPCRs, GABA_BR1 and GABA_BR2 (3–5). GABA_BR1, the first receptor to be cloned, was found to bind GABA with low affinity and couple much less efficiently to effectors than native GABA_B receptors (6). It was soon established that GABA_BR1, when expressed alone in heterologous systems, could not traffic efficiently to the cell surface but was rather retained in the endoplasmic reticulum (ER) due to the presence of an ER retention motif on its intracellular C terminus (7, 8). A second receptor, GABA_BR2, was subsequently cloned and found to be capable of trafficking to the cell surface by itself yet incapable of binding ligand or coupling to G proteins. When GABA_BR1 and GABA_BR2 were co-expressed in heterologous cells, they were found to form functional surface-expressed receptors with properties similar to those of some native GABA_B receptors (9–11). In the heterodimer, GABA_BR1 is thought to bind the ligand (12), whereas GABA_BR2 is believed to be the primary G protein contact site (13–15).

Despite the recent advances in the understanding of GABA_B receptors at the molecular level, several puzzling facts remain. First, despite the apparent functional requirement for heterodimerization, GABA_BR1 is distributed in many regions of the brain (for example, the anterior pituitary and interneurons of the hippocampus) and periphery (uterus, spleen) that show no GABA_BR2 expression but have demonstrable GABA binding and responses (16–18). Second, native GABA_B receptors exhibit tremendous ligand binding heterogeneity, which splice variants of the cloned receptors GABA_BR1 and GABA_BR2 do not adequately explain (4, 19–22). Finally, numerous reports suggest that GABA_B receptors participate in physiological cross-talk with other receptors through unknown mechanisms. Of particular note are functional interactions between GABA_A and GABA_B receptors in regulating each other's binding properties (23–25) and activity (24, 26–29).

In recent years, heterodimerization of receptors has helped to explain some examples of pharmacological heterogeneity and cross-talk between other neurotransmitter receptors (30, 31). For example, heterodimerization of κ and δ opioid receptors results in a new receptor with distinct pharmacological properties (32). Furthermore, physiologically important heterodimerization has been demonstrated not only between GPCRs, but also between GPCRs and ionotropic receptors. For example, GABA_A receptors have been shown to physically interact with dopamine receptor 5 (33), whereas NMDA-type glutamate receptors have been found to associate with dopamine receptor 1 (34, 35), leading to mutual regulation of receptor function.

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¹ The abbreviations used are: GABA, γ -aminobutyric acid; GABA_AR, GABA_A receptor; GABA_BR1, GABA_B receptor 1; GABA_BR2, GABA_B receptor 2; GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; NMDA, N-methyl-D-aspartate; γ 2S, γ 2 short; γ 2L, γ 2 long; HA, hemagglutinin; HEK-293, human embryonic kidney 293 cells; mGluR, metabotropic glutamate receptor; LPA, lysophosphatidic acid receptor; H, histamine receptor; GST, glutathione S-transferase; CT, C terminus; ICL, intracellular loops; ERK, extracellular-signal-regulated kinase; GIRK, G protein-activated inwardly rectifying potassium channel; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

We hypothesized that GABA_BR1 might potentially associate with other receptors and that this might account in part for certain aspects of GABA_B receptor function that are not presently understood. This idea led us to screen a library of different receptors as possible trafficking partners for GABA_BR1. Surprisingly, these screens revealed that co-expression with the γ 2S subunit of the GABA_A receptor produced robust cell surface expression of GABA_BR1 in the absence of GABA_BR2. Furthermore, we found that there is a physical interaction between discrete regions of GABA_BR1 and the GABA_A receptor γ 2S subunit, and that this association has significant functional consequences for GABA_B receptor trafficking and endocytosis.

MATERIALS AND METHODS

Plasmids—Epitope-tagged (HA-, FLAG-, Myc-, and His-tagged) versions of human GABA_BR1b and GABA_BR2 in the mammalian expression vector pcDNA3.1 were kindly provided by Fiona Marshall (Glaxo-SmithKline). β_1 - and β_2 -adrenergic receptor constructs were kindly provided by Robert Lefkowitz (Duke University Medical Center). α_{1A} -, α_{1B} -, and α_{1D} -adrenergic receptor constructs were kindly provided by Ken Minneman (Emory University School of Medicine). α_{2A} -, α_{2B} -, and α_{2C} -adrenergic receptor constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center). Dopamine receptor 1 and 2 constructs were kindly provided by David Sibley (National Institutes of Health). The serotonin 5HT_{1A} receptor construct was kindly provided by John Raymond (Medical University of South Carolina). Angiotensin AT₁ and AT₂ receptor constructs were kindly provided by Victor Dzau (Harvard Medical School). Muscarinic m1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). Opioid receptor constructs (μ , δ , and κ) were kindly provided by Lakshmi Devi (New York University School of Medicine) and Ping-Yee Law (University of Minnesota Medical School). Lysophosphatidic acid-1 and -2 receptor constructs were kindly provided by Jerold Chun (University of California, San Diego). Histamine H₁–4 receptor constructs were kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute). Metabotropic glutamate receptor constructs (mGluR1–8) were kindly provided by Jeff Conn (Emory University School of Medicine). NMDA receptors 1A and 2A constructs were kindly provided by Steve Traynelis (Emory University School of Medicine). GABA_A receptor α 1, β 2, γ 2S, and γ 2L subunit constructs were kindly provided by Cynthia Czajkowski (University of Wisconsin at Madison) and Susan M. J. Dunn (University of Alberta). GIRK1 and GIRK4 constructs were kindly provided by David Mott (Emory University).

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from Invitrogen. HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO₂ incubator. For heterologous expression of receptors, 2–4 μ g of cDNA was mixed with LipofectAMINE (15 μ l), and Plus reagent (10 μ l) (Invitrogen) and added to 5 ml of serum-free medium in 10-cm tissue culture plates containing cells at 60–80% confluency. Following a 4-h incubation, 6 ml of fresh complete medium was added. After another 12–16 h incubation, the medium was changed again, and the cells were harvested 24 h later.

Western Blotting—Samples (5 μ g per lane) were run on 4–20% SDS-PAGE (Invitrogen) for 1 h at 180 V and then transferred to nitrocellulose. The blots were blocked in "blot buffer" (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) for at least 30 min and then incubated with an appropriate primary antibody in blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml of blot buffer and incubated for 1 h at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences and Chemicon) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Biosciences or Pierce.

Antibodies—The primary antibodies utilized were 12CA5 monoclonal anti-HA antibody (Roche Applied Science), M2 monoclonal anti-FLAG antibody (Sigma), monoclonal anti-c-Myc 9E10 antibody (Sigma), anti-GABA_BR1 antibody, anti-GABA_BR2 antibody (Chemicon), anti-GABA_A α 1 antibody, anti-GABA_A β 2 antibody (Upstate Biotechnology), and anti-GABA_A γ 2 antibody (Alpha Diagnostic International).

Surface Expression Assay—HA-GABA_BR1 cDNA was transfected either alone or with other receptor cDNAs. Transfected HEK-293 cells

were plated in 35-mm tissue culture plates at 80% confluency. 24 h later, the cells were fixed in 4% paraformaldehyde in PBS/Ca²⁺ for 30 min and then blocked with "cell blocking buffer" (2% non-fat dry milk in PBS/Ca²⁺) for 30 min at room temperature. They were then incubated with a monoclonal 12CA5 anti-HA antibody for 1 h to detect the HA-GABA_BR1 on the cell surface. Following three washes (5 min) with cell blocking buffer, the cells were incubated with horseradish peroxidase-coupled anti-mouse secondary antibody (Amersham Biosciences) for 1 h. The cells were washed three times for 5 min with cell blocking buffer, twice with PBS, and then developed with 2 ml of enzyme-linked immunosorbent assay SuperSignal Pico ECL from Pierce for exactly 15 s. Chemiluminescence of the whole 35-mm plate was quantified in a TD20/20 luminometer (Turner Designs). For each data point, 3–5 dishes were averaged per experiment. The results were analyzed using one-way analysis of variance and Dunnett's post hoc tests where applicable (GraphPad Prism). For each transfection condition in each surface expression experiment, matching dishes of transfected cells were harvested and examined via Western blot to confirm the expression of the various receptors involved.

Double Immunofluorescence Microscopy—HEK-293 cells were transiently transfected with HA-GABA_BR1 alone, HA-GABA_BR1/FLAG-GABA_BR2, or HA-GABA_BR1/Myc-GABA_A γ 2S. Transfected cells were plated in slides, fixed with 4% paraformaldehyde, and permeabilized with "saponin buffer" containing 2% bovine serum albumin and 0.04% saponin in PBS for 30 min at room temperature. The cells were then incubated with M2 monoclonal anti-FLAG antibody (Sigma), monoclonal anti-c-Myc 9E10 antibody (Sigma), and anti-GABA_BR1 antibody (Chemicon) for 1 h at room temperature. After three washes (1 min) with saponin buffer, the cells were incubated with a rhodamine red-conjugated anti-mouse IgG at 1:200 dilution and FITC-conjugated anti-guinea pig IgG at 1:200 dilution (Jackson ImmunoResearch) for 1 h at room temperature. After three washes (1 min) with saponin buffer and one wash with PBS, coverslips were mounted, and rhodamine red-labeled FLAG-GABA_BR2 or Myc-GABA_A γ 2S and FITC-labeled HA-GABA_BR1 were visualized with a Zeiss LSM-510 laser confocal microscope. Multiple control experiments, utilizing either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody, revealed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in the transfected cells was specific.

Immunoprecipitation—Cells were harvested and lysed in 500 μ l of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, and the protease inhibitor mixture from Roche Applied Science). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 4–8 μ g of respective antibody and 50 μ l of protein A/G-agarose beads (Oncogene) or 50 μ l of beads covalently linked to anti-FLAG antibodies (Sigma) or anti-HA antibodies (Covance) for 2 h with end-over-end rotation at 4 °C. After five washes with 1 ml of lysis buffer, the immunoprecipitated proteins were eluted from the beads with 1 \times SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to Western blot analyses.

GST Pull-down Assay—GABA_BR1 C terminus and truncations were prepared with a hexahistidine C-terminal tag via PCR amplification of full-length rat GABA_BR1 and subcloned into the pGEX-4T1 vector (Amersham Biosciences) using EcoRI and XhoI restriction enzymes. GST α 1 ICL and GST β 2 ICL were generous gifts from Cynthia Czajkowski (University of Wisconsin, Madison). GST γ 2S ICL containing 86 amino acids was PCR-amplified from full-length γ 2S construct and subcloned into pGEX-4T1 vector using EcoRI and XhoI restriction enzymes. Fusion proteins grown in *E. coli* were isolated and purified on glutathione-agarose beads. Aliquots of the fusion protein on beads were blocked 30 min with 1 ml of "BSA buffer" (3% BSA, 10 mM HEPES, 50 mM NaCl, 0.1% Tween 20) at 4 °C. Solubilized brain lysates or lysates from transfected HEK-293 cells were then incubated with the beads end-over-end at 4 °C overnight. Following three washes with 1 ml of BSA buffer, the proteins were eluted off the beads with sample buffer, resolved on SDS-PAGE gels, and analyzed via Western blot using appropriate antibodies.

ERK Activation Assay—HEK-293 cells were transfected with HA-GABA_BR1 alone, HA-GABA_BR1/FLAG-GABA_BR2, HA-GABA_BR1/Myc-GABA_A γ 2S, or HA-GABA_BR1/GABA_A α 1 β 2 γ 2S. Transfected HEK-293 cells were plated in 35-mm tissue culture plates at 80% confluency and serum-starved overnight the day before the assay. The cells were stimulated with 100 μ M baclofen, rinsed with ice-cold PBS/Ca²⁺, and lysed

in 150 μ l of sample buffer. The cell lysates were run on SDS-PAGE gels and then analyzed via Western blotting with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies (Cell Signaling).

Electrophysiology—Stage V–VI oocytes were harvested from *Xenopus laevis* and prepared for injection. Single oocytes were injected within 24 h with 5 nl of cRNA prepared from cDNA using RNA Express kit (Ambion) (1 ng to 1 μ g/ μ l per subunit) and were assayed functionally at least 2 days after cRNA injection. GABA_A receptor-mediated currents were measured from oocytes perfused with "ND96/Ca²⁺" (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.2) in a recording chamber. Recordings were made using 2-electrode voltage clamp while holding the oocytes at -80 mV with borosilicate electrodes filled with 300 mM KCl. GABA_A receptor currents were measured by perfusing GABA (Tocris) or GABA plus diazepam (Sigma) directly onto oocytes under clamp conditions. GIRK current measurement was made in an identical set up, but the recordings were made in "40K solution" (60 mM NaCl, 40 mM KCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 6 mM HEPES, pH 7.4), and oocytes were clamped at a voltage of -100 mV. GABA (Tocris) or baclofen (Sigma) was dissolved in 40K solution and applied by bath superfusion.

Electrophysiology Analysis—The current peaks were measured using mini-analysis (Synaptosoft) and analyzed with GraphPad Prism. All concentration-response data were fit by Equation 1,

$$I = I_{\max} \times [L]^n / ([L]^n + [EC_{50}]^n) \quad (\text{Eq. 1})$$

where I is the current response; I_{\max} is the maximal current response; $[L]$ is the drug concentration; EC_{50} is the drug concentration that evokes half-maximal current response; and n is the Hill coefficient. The diazepam potentiation of I_{GABA} was defined as shown in Equation 2,

$$P = [I_{\text{GABA} + \text{diazepam}} / I_{\text{GABA}}] - 1 \quad (\text{Eq. 2})$$

where $I_{\text{GABA} + \text{diazepam}}$ is the current response in the presence of GABA and diazepam, and I_{GABA} is the current evoked solely by GABA. Diazepam potentiation was measured at a low concentration (10 μ M) of GABA ($EC_{50} \sim EC_{10}$). The results were statistically compared using unpaired t tests as required.

Internalization Assay—HEK-293 cells were transfected with HA-GABA_BR1/FLAG-GABA_BR2 or HA-GABA_BR1/FLAG-GABA_BR2 + Myc-GABA_A γ 2S. Transfected cells were plated in 35-mm tissue culture plates at 80% confluency. On the day of the assay, the cells were stabilized at room temperature for 2 h and stimulated with 100 μ M GABA for 30 min at room temperature, and the amount of GABA_BR1 on the cell surface was assayed in a manner identical to the surface expression assay described above.

RESULTS

The GABA_A Receptor γ 2S Subunit Promotes GABA_BR1 Cell Surface Expression—To screen for potential interacting partners that might aid in trafficking GABA_BR1 to the plasma membrane, we used a cell surface expression assay that has been used previously to study GABA_BR1 plasma membrane expression (7). HA-tagged GABA_BR1 was sequentially co-expressed with various GPCRs and ionotropic receptors in HEK-293 cells, and the cell surface expression of GABA_BR1 was analyzed using a luminometer-based assay. In this assay, GABA_BR1 expressed alone showed barely detectable cell surface expression, whereas co-expression with GABA_BR2 yielded a nearly 40-fold increase in GABA_BR1 cell surface expression, as reported previously (3, 7). GABA_BR1 was also co-expressed in these screens with 34 other GPCRs. In contrast to GABA_BR2, none of these receptors significantly facilitated GABA_BR1 surface expression. It has been reported previously that co-expression with the metabotropic glutamate receptor mGluR4 may (36) or may not (8) have a modest effect on GABA_BR1 surface expression, but our studies consistently revealed no effect of any of the mGluR subtypes on trafficking of GABA_BR1 to the cell surface. Similarly, co-expression with the NMDA-type glutamate receptor subunits NR1 and -2A also failed to enhance the surface expression of GABA_BR1. Strikingly, however, co-expression with either the γ 2S subunit of the GABA_A receptor or the entire GABA_A receptor complex composed of α 1, β 2, and γ 2S subunits increased the surface expres-

sion of GABA_BR1 by \sim 15–20-fold (Fig. 1). Because co-expression with the α 1 and β 2 subunits alone had no effect on GABA_BR1 surface expression, these results revealed that the γ 2S subunit is capable of trafficking GABA_BR1 to the cell surface in the absence of GABA_BR2.

The surprising effect of γ 2S subunit co-expression on GABA_BR1 plasma membrane trafficking was confirmed via immunocytochemistry. HEK-293 cells were transfected with either (i) GABA_BR1 alone, (ii) differentially tagged GABA_BR1 and GABA_BR2, or (iii) differentially tagged GABA_BR1 and GABA_A receptor γ 2S subunit. The receptors were labeled with specific fluorescent antibodies and visualized via confocal microscopy. These co-localization studies revealed that GABA_BR1 was diffusely distributed throughout the cytoplasm when expressed alone (Fig. 2, A–C) but found predominantly at the plasma membrane when co-expressed with GABA_BR2 (Fig. 2, D–F). Remarkably, a similar localization of GABA_BR1 to the plasma membrane was observed following co-expression with the γ 2S subunit (Fig. 2, G–I). These data confirm that the GABA_A receptor γ 2S subunit can mimic GABA_BR2 in its ability to promote the trafficking of GABA_BR1 to the cell surface and furthermore show that the γ 2S subunit and GABA_BR1 are co-localized in the plasma membrane when co-expressed in HEK-293 cells.

GABA_B Receptors and GABA_A Receptor Subunits Physically Associate in Transfected Cells and Native Brain Tissue—The profound enhancement of GABA_BR1 surface expression induced by co-expression with the GABA_A receptor γ 2S subunit suggested that there might be a physical interaction between these two proteins. This possibility was examined via co-immunoprecipitation experiments. As shown in Fig. 3A, GABA_BR1 was robustly co-immunoprecipitated with the γ 2S subunit from transfected HEK-293 cell lysates, revealing that the two proteins can indeed associate in a cellular context. The potential interactions of other GABA_A receptor subunits with GABA_BR1 were also examined. It was found that GABA_BR1 could be co-immunoprecipitated from transfected HEK-293 cell lysates with α 1 subunits (Fig. 3B) but not with β 2 subunits (Fig. 3C). Moreover, GABA_BR1 was still found to associate with γ 2S and α 1 subunits even in the presence of GABA_BR2. Similarly, GABA_BR2 could be co-immunoprecipitated with γ 2S and α 1 subunits but not with β 2 subunits (Fig. 3, A–C, bottom panels) both in the presence and absence of GABA_BR1. We also conducted reverse experiments to see whether GABA_A receptors were co-immunoprecipitated with GABA_BR1. As shown in Fig. 3D, immunoprecipitation of FLAG-GABA_BR1 from cells transfected with α 1 β 2 γ 2S resulted in robust co-immunoprecipitation of all of the GABA_A receptor subunits. The extent of α 1 subunit co-immunoprecipitation with GABA_BR1 was similar for α 1 alone (10th lane) versus α 1 β 2 (9th lane) versus α 1 β 2 γ 2S (8th lane). These data suggest that both GABA_BR1 and GABA_BR2 can associate with functional GABA_A receptor pentamers as well as with the individual subunits α 1 and γ 2S. Finally, we examined the potential association of endogenous GABA_B receptors and GABA_A receptor γ 2 subunits in native brain tissue. As shown in Fig. 3E, GABA_B receptors were strongly co-immunoprecipitated with γ 2 subunits from solubilized rat brain lysates, demonstrating that GABA_B receptors and GABA_A receptor γ 2 subunits form complexes not only in transfected cells but also in native brain tissue.

GABA_BR1 Surface Expression Is Promoted by γ 2S but Not α 1 β 2 or γ 2L Subunits—The co-immunoprecipitation experiments revealed that both α 1 and γ 2S subunits can associate with GABA_BR1. These findings were somewhat surprising, because our initial screens had indicated that the α 1 subunit was unable to promote GABA_BR1 trafficking to the plasma

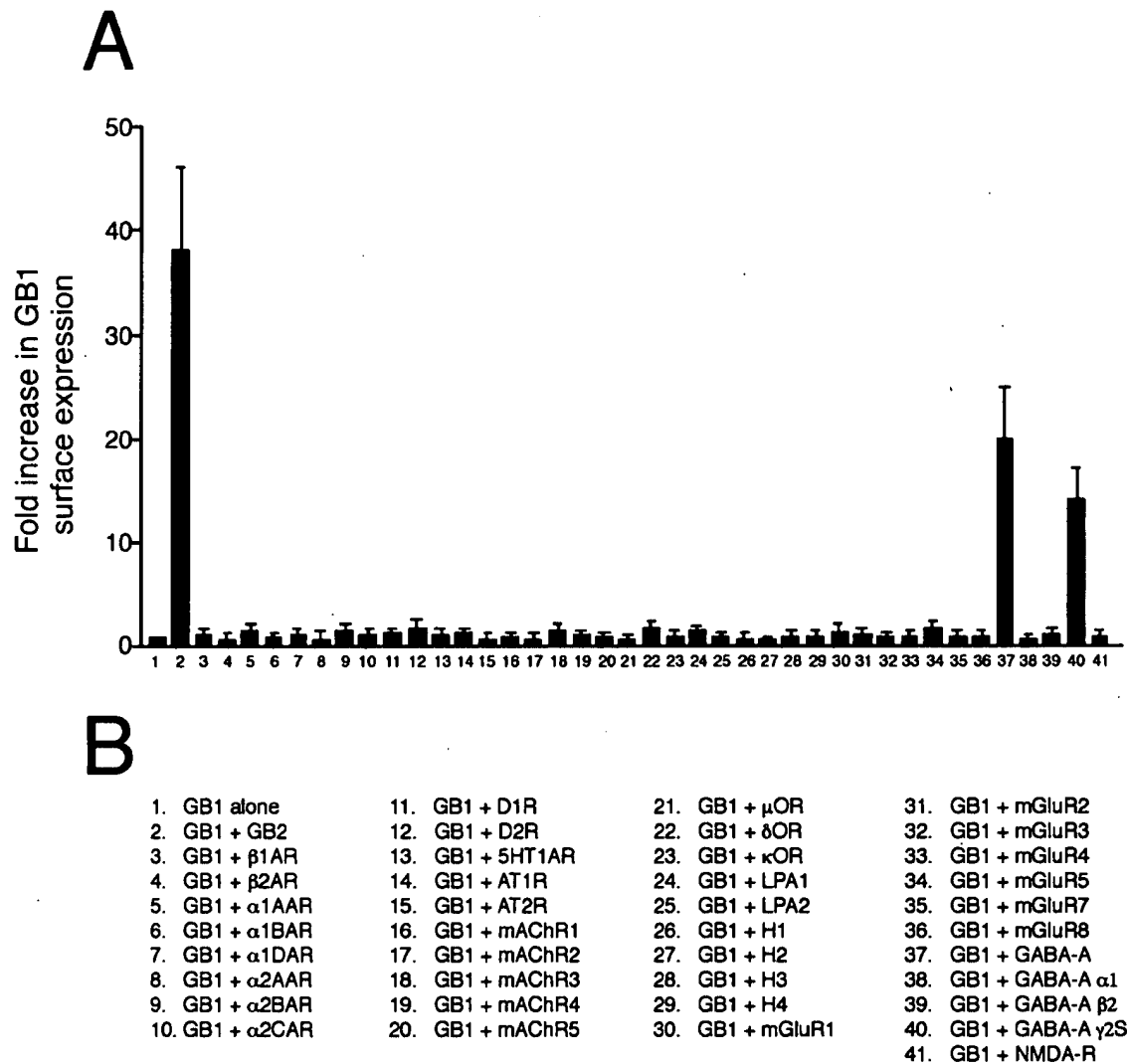


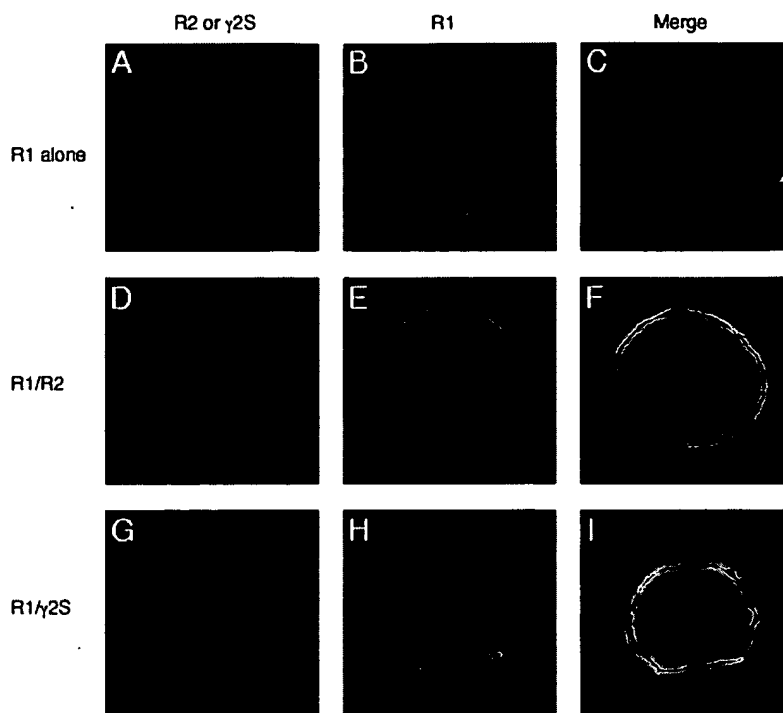
FIG. 1. GABA_B1 is trafficked to the cell surface by GABA_Aγ2S but not by other receptors. HA-GABA_B1 surface expression was detected and quantified by a luminometer-based assay (A) following HA-GABA_B1 co-expression in HEK-293 cells with other GPCRs and ligand-gated ion channels listed as 1–41 (B). The receptors examined in this study were chosen because they are known to be expressed in at least some of the same brain regions as GABA_B1. In these experiments, GABA_B1 expressed by itself was barely detectable on the cell surface, whereas GABA_B1 co-expressed with GABA_B2 as a positive control showed a nearly 40-fold increase in surface expression over GABA_B1 alone. Co-expression with most of the other receptors examined had no significant effect on GABA_B1 surface expression, but co-expression of GABA_B1 with the GABA_A receptor γ2S subunit or with the whole GABA_A receptor complex containing the γ2S subunit showed a 15–20-fold increase in surface expression. The bars and error bars represent mean ± S.E. for 3–5 independent experiments. Abbreviations are as follows: GB, GABA_B receptor; AR, adrenergic receptor; DR, dopamine receptor; 5HT₁AR, serotonin receptor; ATR, angiotensin receptor; mAChR, muscarinic acetylcholine receptor; OR, opioid receptor; LPA, lysophosphatidic acid receptor; H, histamine receptor; mGluR, metabotropic glutamate receptor.

membrane. One potential explanation for this result could be that the α1 subunit cannot traffic GABA_B1 to the cell surface because it cannot access the cell surface by itself. It has been shown that the α1 and β2 subunits expressed individually in HEK-293 cells are retained in the endoplasmic reticulum, whereas α1 and β2 subunits expressed together form functional channels on the cell surface (37). Hence, we investigated the ability of α1β2 receptors to traffic GABA_B1 to the cell surface. We found, however, that GABA_B1 exhibited no significant increase in trafficking to the plasma membrane when co-expressed with α1β2 receptors (Fig. 4A). In contrast, co-expression of GABA_B1 with the α1β2γ2S GABA_A receptor resulted in a more than 20-fold enhancement in GABA_B1 surface expression, as shown earlier. In matching control experiments, we found that GABA_A receptor α1 subunits were efficiently trafficked to the cell surface when expressed with β2 subunits but not when expressed alone (data not shown), consistent with previous observations (37). These data reveal that

α1 and β2 subunits do not promote GABA_B1 cell surface expression and that the γ2S subunit is required for this effect.

There are two known splice variants of the γ2 subunit, γ2S and γ2L, which differ in that γ2L has an additional 8 amino acids on one of its intracellular loops. We examined the ability of these two splice variants to regulate GABA_B1 subcellular localization. As shown earlier, co-expression of GABA_B1 with the γ2S subunit resulted in a more than 15-fold increase in GABA_B1 surface expression. In contrast, co-expression of GABA_B1 with the γ2L subunit had absolutely no effect at all on the amount of GABA_B1 trafficked to the cell surface (Fig. 4B). This may be attributed to the fact that the γ2S subunit can traffic to the cell surface when expressed alone in heterologous cells, although it does not form functional receptors (38). The γ2L subunit, conversely, is retained in the endoplasmic reticulum when expressed alone and is trafficked to the cell surface only when expressed with α and β subunits to form fully functional GABA_A receptors (38). These findings demonstrate that

FIG. 2. Co-expression of GABA_BR1 with the GABA_A receptor γ 2S subunit results in plasma membrane expression and co-localization of GABA_BR1/ γ 2S. HA-GABA_BR1 expressed alone in HEK cells and visualized with a FITC-conjugated antibody exhibited diffuse intracellular staining (A–C). When HA-GABA_BR1 (FITC) was expressed with FLAG-GABA_BR2, which was visualized with rhodamine red, it was concentrated at the plasma membrane along with GABA_BR2 (D–F). Similarly, co-expression of HA-GABA_BR1 (FITC) with Myc-GABA_A γ 2S (rhodamine red) also resulted in striking co-localization and membrane targeting of both receptors (G–I). The data presented here are representative of three independent experiments.



the two GABA_A receptor γ 2 subunit splice variants exert radically different effects on GABA_BR1 trafficking, with only γ 2S being competent to promote GABA_BR1 cell surface expression.

The C terminus of GABA_BR1 Is Sufficient but Not Necessary for Association with GABA_A Receptors—In order to elucidate the structural determinants of the interaction between GABA_BR1 and GABA_A receptor subunits, we constructed a glutathione *S*-transferase (GST) fusion protein corresponding to the GABA_BR1 C terminus (CT), which is the largest cytoplasmic domain of the receptor (amino acids 856–960 of the full-length polypeptide). We then assessed the ability of this fusion protein to interact physically with full-length GABA_A receptor subunits. The GABA_BR1-CT was able to pull down GABA_A receptor subunits from solubilized rat brain lysates as detected by Western blotting with an antibody against the γ 2 subunit (Fig. 5A, 1st 3 lanes). Conversely, GST alone did not detectably pull down any GABA_A receptor immunoreactivity. These data indicate that the C terminus of GABA_BR1 is sufficient to mediate an interaction with GABA_A receptors. In order to pinpoint the exact region involved, GST fusion proteins representing three truncations of the C terminus of GABA_BR1 from the N-terminal end, corresponding to amino acids 879–960, 907–960, and 934–960, were prepared, and the ability of these truncants to pull down the GABA_A receptor from solubilized brain lysates was examined. In these experiments, the two longest GABA_BR1-CT fusion proteins were capable of pulling down the GABA_A receptor γ 2 subunit, whereas the shortest fusion protein was not (Fig. 5A, last 3 lanes). These data indicated that the amino acids mediating the association are located between residues 907 and 934 of GABA_BR1. GST fusion proteins of five incremental truncations made between the region encompassed by amino acids 907–960 were then analyzed in a similar pull-down assay. In this experiment, addition of seven amino acids to the N terminus of the shortest fusion protein (934–960) of GABA_BR1 conferred the ability to pull down the GABA_A γ 2 subunit. Thus, these studies defined a region of seven amino acids in the GABA_BR1 C terminus (PPTPPDP) as a key determinant of the association with the GABA_A receptor γ 2 subunit (Fig. 5B).

In order to assess the requirement of the PPTPPDP motif for mediating the interaction between GABA_BR1 and the γ 2S subunit, we prepared a mutant version of GABA_BR1 with the N terminus and the seven-transmembrane region intact but the C terminus truncated to remove the PPTPPDP motif and most of the GABA_BR1-CT. This truncated receptor was found to co-immunoprecipitate the GABA_A receptor almost as well as wild-type GABA_BR1 (Fig. 5C). These findings suggest that the GABA_BR1 C terminus is sufficient but not necessary for interaction with the γ 2S subunit and that other regions of GABA_BR1, such as perhaps the transmembrane domains, must be involved in the interaction. Similar results have been found for the interaction of GABA_BR1 with GABA_BR2, where the C termini of the receptors are clearly involved in the interaction but are by no means required (7, 8, 39).

Because the pull-down studies revealed that the intracellular C terminus of GABA_BR1 was sufficient for interaction with the γ 2S subunit, we examined the intracellular regions of the GABA_A receptor subunits for their ability to interact with GABA_BR1. GST fusion proteins corresponding to the intracellular loop (ICL) region between transmembrane 3 and transmembrane 4 of the α 1, β 2, and γ 2S subunits were prepared and analyzed in a pull-down assay performed with lysates from cells transfected with GABA_BR1. As shown in Fig. 5D, the γ 2S subunit ICL was capable of pulling down GABA_BR1, whereas GST alone, the α 1 subunit ICL, and the β 2 subunit ICL did not detectably pull down any GABA_BR1 immunoreactivity. These data demonstrate that the intracellular loop of the γ 2S subunit is sufficient to mediate interaction with GABA_BR1.

Functional Consequences of Hetero-oligomerization between GABA_A and GABA_B Receptors—We hypothesized that the interaction between GABA_BR1 and the GABA_A receptor γ 2S subunit might serve as a point of cross-talk between GABA_A and GABA_B receptors and facilitate the mutual regulation of the receptors. To test this idea, we started by examining the responsiveness of the GABA_A receptor channel in *Xenopus* oocytes to GABA and benzodiazepines. Benzodiazepines are allosteric modulators of the GABA_A receptor that increase the frequency of channel opening in the presence of GABA by binding to a

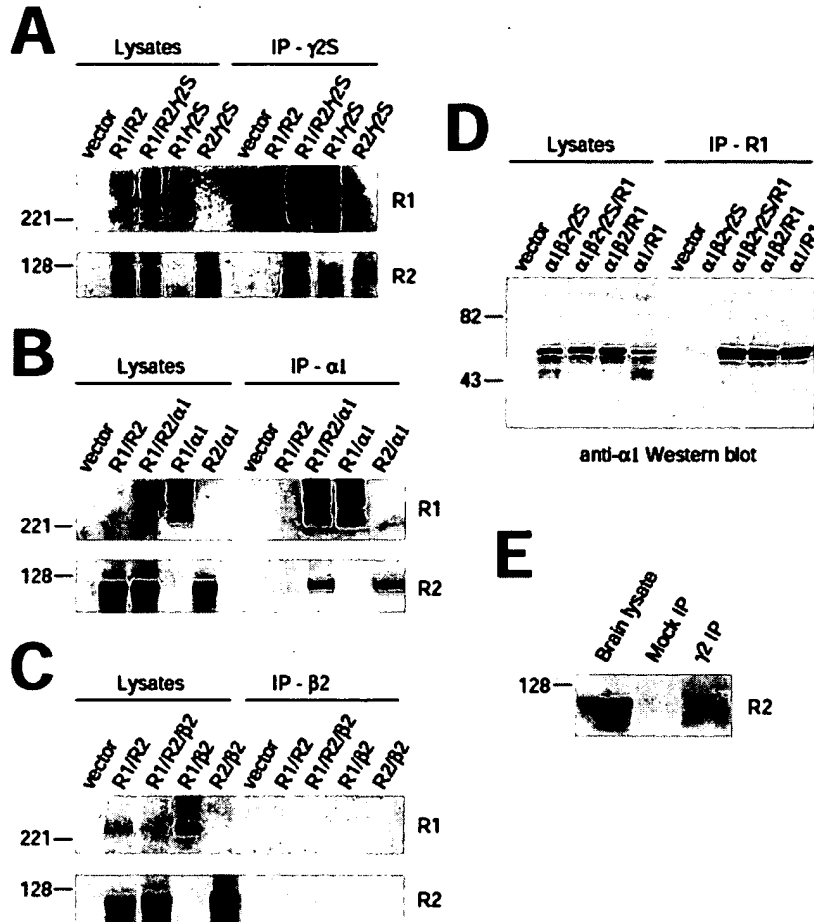


FIG. 3. Co-immunoprecipitation of GABA_A and GABA_B receptors from transfected cells and native brain tissue. A, HEK-293 cells were transfected with the following combinations of receptors: empty vector, GABA_BR1/GABA_BR2, GABA_BR1/GABA_BR2 plus Myc-GABA_Aγ2S, GABA_BR1 plus Myc-GABA_Aγ2S, and GABA_BR2 plus Myc-GABA_Aγ2S. Cells were harvested, solubilized, and immunoprecipitated (IP) using an anti-Myc antibody. Western blot analysis revealed robust co-immunoprecipitation of GABA_BR1 and GABA_BR2 with the GABA_A receptor γ2S subunit (last 3 lanes). GABA_BR1 immunoreactivity was usually observed as a higher molecular weight oligomer, as reported previously (9). B, HEK-293 cells were transfected with the same combinations of receptors as shown in A, except that Myc-GABA_Aγ2S was replaced with FLAG-GABA_Aα1. Western blot analysis following immunoprecipitation with anti-FLAG antibodies revealed that GABA_BR1 and GABA_BR2 co-immunoprecipitated with the GABA_Aα1 subunit (last 3 lanes). C, HEK-293 cells were transfected with the same combinations of receptors as shown in A except that Myc-GABA_Aγ2S was replaced with GABA_Aβ2 and immunoprecipitated with β2/3 antibody. However, neither GABA_BR1 nor GABA_BR2 was found to co-immunoprecipitate with the GABA_Aβ2 subunit (last 3 lanes). D, GABA_BR1 associates equally well with GABA_A receptor subunits expressed individually or together. HEK-293 cells were transfected with either empty vector, α1β2γ2S, FLAG-GABA_BR1 plus GABA_Aα1β2γ2S, FLAG-GABA_BR1 plus GABA_Aα1β2 only, or FLAG-GABA_BR1 plus GABA_Aα1 only. GABA_BR1 was immunoprecipitated with anti-FLAG antibody, and the co-immunoprecipitation of the GABA_A receptor subunits was assessed. All three subunits were robustly co-immunoprecipitated (α1 is shown; β2 and γ2S are not shown), and the extent of α1 subunit co-immunoprecipitation with GABA_BR1 was consistently unchanged by the presence or absence of the other GABA_A receptor subunits. E, GABA_B receptors associate with GABA_A receptor γ2 subunits in native brain tissue. Solubilized rat brain lysates were subjected to immunoprecipitation with either γ2 antibody (γ2 IP) or no antibody (Mock IP). Both GABA_BR1 (not shown) and GABA_BR2 were found to be specifically immunoprecipitated by the γ2 antibody. The data presented in all of the panels of this figure are representative of three to five independent experiments each.

unique site on the receptor protein (2). It is well established that the α and γ subunits confer benzodiazepine binding properties to GABA_A receptors by forming a binding pocket at their interface (40). As both γ and α subunits were found in our studies to be involved in the association between GABA_A and GABA_B receptors, we monitored GABA_A receptor currents and benzodiazepine responsiveness in the presence and absence of GABA_BR1 co-expression. Concentration response curves were constructed by measuring currents evoked by a low, non-saturating dose of GABA (10 μM) in the presence of varying concentrations of diazepam, a classical benzodiazepine. No significant differences in EC₅₀ values for diazepam were found between GABA_A receptors alone (α1β2γ2S; EC₅₀ = 30.4 ± 1.7 nM, *n* = 5) and GABA_A receptors co-expressed with GABA_BR1 (EC₅₀ = 27.9 ± 6.2 nM, *n* = 9) (Fig. 6A). Thus, we found no evidence that co-expression with GABA_BR1 alters the sensitivity of GABA_A receptors to diazepam modulation. We also examined whether

co-expression with GABA_BR1 might affect the activation of GABA_A channels by GABA. In these studies, a modest increase in GABA potency was observed for GABA_A receptors co-expressed with GABA_BR1 (EC₅₀ = 11.1 ± 1.7 μM, *n* = 10) compared with GABA_A receptors expressed alone (EC₅₀ = 18.7 ± 1.5 μM, *n* = 8) (Fig. 6B). These data suggest that agonist activation of GABA_A receptors can be subtly modulated by the association of the receptors with GABA_B receptors.

In addition to studying the effects of GABA_B receptor co-expression on GABA_A receptor function, we also examined the consequences of GABA_A receptor co-expression on the function of GABA_B receptors. It has been reported that GABA_BR1 is expressed at high levels in certain regions that lack detectable GABA_BR2 but have measurable responses to GABA (18). Thus, we explored the possibility that the GABA_BR1/γ2S complex might be functional in the absence of GABA_BR2. Two different types of experiments were conducted to address this question.

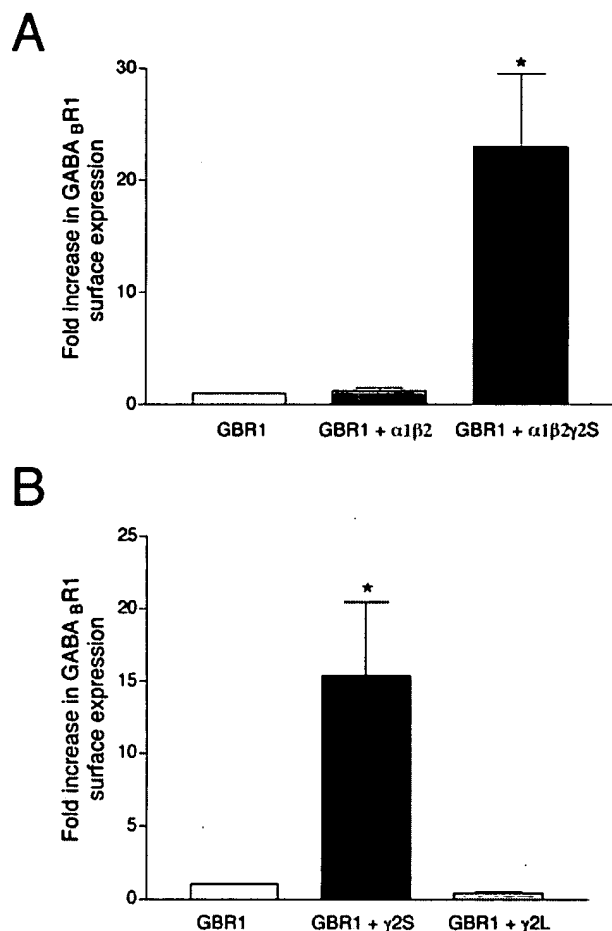


FIG. 4. The $\gamma 2S$ subunit, but not other GABA_A receptor subunits, can traffic GABA_B R1 to the cell surface. A, GABA_B R1 surface expression in HEK-293 cells was assessed in the absence and presence of co-transfection with GABA_A receptor $\alpha 1\beta 2$ or $\alpha 1\beta 2\gamma 2S$ subunits. Co-expression with $\alpha 1\beta 2$ was found to be incapable of trafficking GABA_B R1 to the cell surface, whereas co-expression with $\alpha 1\beta 2\gamma 2S$ strongly promoted the cell surface expression of GABA_B R1 (* = $p < 0.05$). B, the two $\gamma 2$ subunit splice variants differ in their ability to traffic GABA_B R1 to the cell surface. GABA_B R1 surface expression was assessed in HEK-293 cells in the absence and presence of either $\gamma 2S$ or $\gamma 2L$ subunits. The $\gamma 2S$ splice variant promoted GABA_B R1 surface expression by more than 15-fold (* = $p < 0.05$), whereas the $\gamma 2L$ splice variant had no significant effect on GABA_B R1 surface expression. Expression levels of the two splice variants in these experiments were comparable, as assessed by Western blots (not shown). The data shown in both panels of this figure are representative of three independent experiments.

First, the ability of GABA_B receptors to stimulate extracellular signal-regulated kinase (ERK) activity was measured, because many G_{i/o}-coupled receptors have been shown to activate the ERK pathway via G protein $\beta\gamma$ subunits (41). ERK activation has not been shown yet for GABA_B receptors, however; so we first performed time course studies to look for potential increases in ERK phosphorylation following GABA_B receptor stimulation. HEK-293 cells were transfected with GABA_B receptors (GABA_B R1 and GABA_B R2) and stimulated with 100 μM baclofen, a specific GABA_B receptor agonist, for 1, 2, 5, 10, and 15 min. ERK activation was monitored by probing the cell lysates for phospho-ERK immunoreactivity on Western blots. To control for loading differences, Western blots for total ERK protein were also performed. As shown in Fig. 7A, GABA_B receptor stimulation produced a profound increase in ERK phosphorylation at time points between 2 and 5 min. These data clearly demonstrate that the GABA_B receptors are able to

activate ERK following expression in HEK-293 cells. We next assessed if GABA_B R1 co-expressed with GABA_A receptors was capable of activating ERK in the absence of GABA_B R2. HEK-293 cells were transfected with the following cDNA combinations: empty vector, GABA_B R1 alone, GABA_B R1/GABA_B R2, GABA_B R1/ $\gamma 2S$, or GABA_B R1/ $\alpha 1\beta 2\gamma 2S$. The various sets of transfected cells were then stimulated with 100 μM baclofen for 5 min, and ERK activation was assessed via Western blots. Stimulation of cells transfected with empty vector or GABA_B R1 alone did not activate ERK, whereas stimulation of GABA_B R1/GABA_B R2 resulted in robust activation of ERK, as shown above. However, stimulation of cells transfected with GABA_B R1/ $\gamma 2S$ or GABA_B R1/ $\alpha 1\beta 2\gamma 2S$ did not result in any detectable activation of ERK (Fig. 7B). These data suggest that although GABA_B R1 can be trafficked to the cell surface by co-expression with the GABA_A receptor $\gamma 2S$ subunit, GABA_B R1 cannot stimulate ERK phosphorylation in the absence of GABA_B R2.

In a related set of experiments, we employed oocyte electrophysiology to test the physiological responsiveness of GABA_B R1/ $\gamma 2S$ and GABA_B R1/ $\alpha 1\beta 2\gamma 2S$ complexes. It is well established that GABA_B receptors can activate G protein-activated inwardly rectifying potassium (GIRK) channels in oocytes, whereas GABA_B R1 expressed in the absence of GABA_B R2 cannot independently activate GIRK channels (10). Oocytes were injected with cRNAs encoding GIRK1 and GIRK4 channels along with the following receptor combinations: GABA_B R1 alone, GABA_B R1/GABA_B R2, GABA_B R1/ $\gamma 2S$, or GABA_B R1/ $\alpha 1\beta 2\gamma 2S$. The responsiveness of each group of oocytes to various concentrations of baclofen was then monitored by using 2-electrode voltage clamping. As in the ERK assay, GABA_B R1 alone, GABA_B R1/ $\gamma 2S$, and GABA_B R1/ $\alpha 1\beta 2\gamma 2S$ were incapable of activating GIRK currents even at high agonist concentrations. In contrast, oocytes injected with GABA_B R1/GABA_B R2 exhibited robust GIRK-mediated currents in response to baclofen (Fig. 7C). Thus, two different techniques provided evidence that hetero-oligomerization with GABA_A receptors does not confer G protein-dependent signaling to GABA_B R1 in the absence of GABA_B R2. These observations are consistent with previous reports that have shown GABA_B R2 to be the G protein binding partner of the GABA_B receptor heterodimer (13–15). We extended the oocyte studies to examine the possibility that the $\gamma 2S$ subunit might affect the potency of GABA at GABA_B receptors. However, no significant differences were observed in the potency of GABA for GABA_B receptors (GABA_B R1/GABA_B R2) expressed in the absence ($EC_{50} = 4.5 \pm 0.6 \mu M$, $n = 5$) or presence of the $\gamma 2S$ subunit ($EC_{50} = 4.5 \pm 0.6 \mu M$, $n = 7$) (Fig. 7D).

The $\gamma 2S$ Subunit Confers Agonist-dependent Internalization to GABA_B Receptors—It has been reported that GABA_B receptors do not undergo agonist-dependent endocytosis or desensitization in HEK-293 cells, although they do desensitize to a significant degree in response to agonist stimulation in cerebellar granule neurons (42). GABA_A receptors, on the other hand, are known to undergo constitutive clathrin-mediated endocytosis in both native and transfected cells via association with adaptor protein complex 2 (AP2) adaptins (43, 44). Interestingly, the $\gamma 2S$ subunit has been shown to constantly recycle between the cell surface and the cytoplasm when expressed alone and to also play a key role in GABA_A receptor internalization (38, 43). Since we found that the $\gamma 2S$ subunit associates with GABA_B receptors, we examined whether it might alter the ability of these receptors to undergo endocytosis. Agonist-promoted internalization of GABA_B receptors was studied in HEK-293 cells in the absence and presence of co-expression with the $\gamma 2S$ subunit using the luminometer-based cell surface expres-

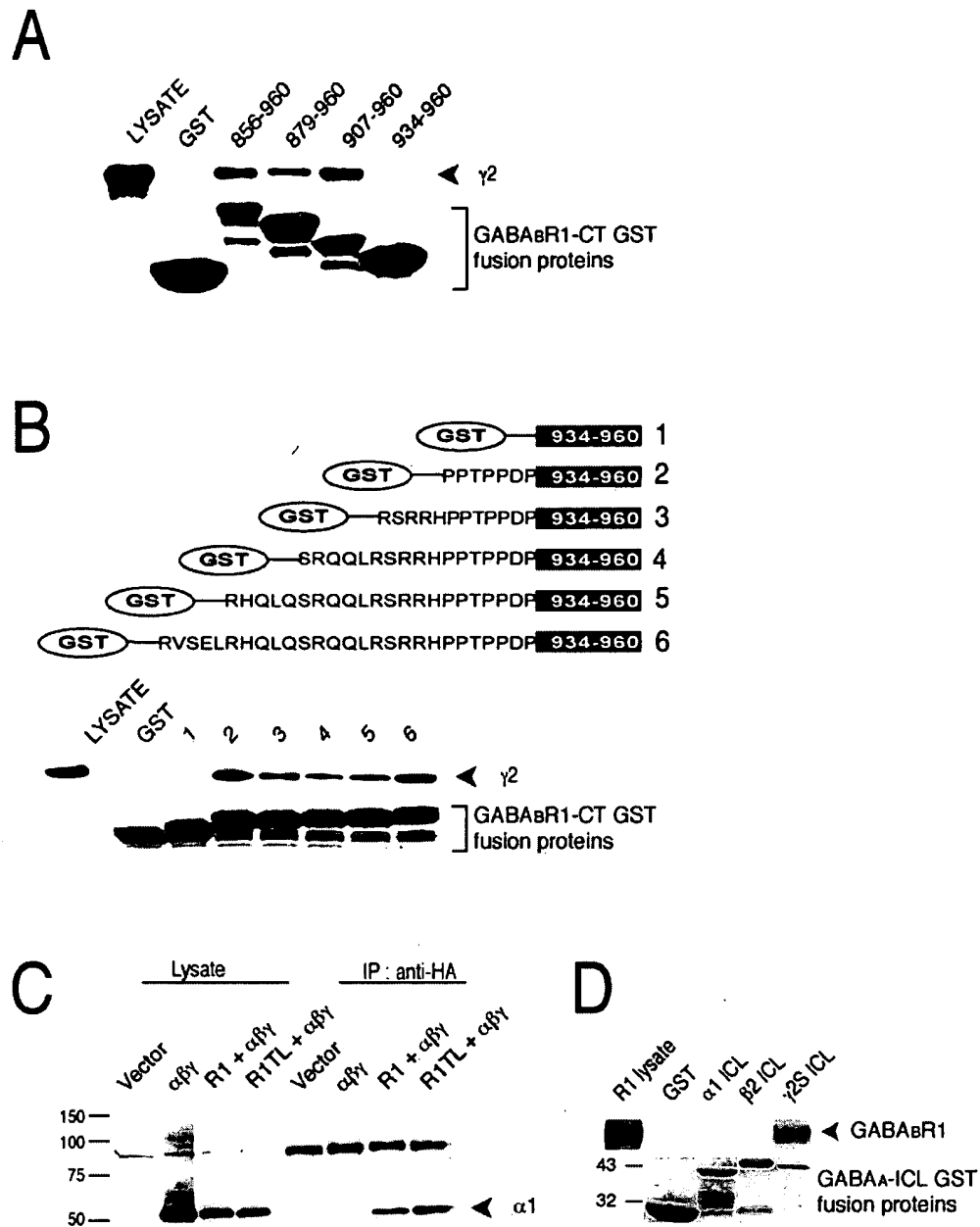


FIG. 5. The GABA_BR1 C terminus is sufficient but not necessary for association with the GABA_A receptor. *A*, discrete region of the GABA_BR1-CT mediates interaction with GABA_A receptors. GST fusion proteins of GABA_BR1-CT (amino acids 856–960) and three truncations made from the N-terminal end, corresponding to amino acids 879–960, 907–960, and 934–960 of the full-length receptor, were incubated with solubilized rat brain lysates and assessed for their ability to pull down GABA_A receptor immunoreactivity. Western blot analyses using an antibody against the γ2 subunit indicated that GABA_A receptors were not pulled down by control GST but were markedly pulled down by the full-length GABA_BR1-CT. Truncations 879–960 and 907–960 also pulled down GABA_A receptor immunoreactivity, whereas truncation 934–960 did not. These data indicate that a key region of interaction lies between amino acids 907–934 of GABA_BR1. The relative sizes and loading levels of the various GABA_BR1-CT-GST truncations are shown in the Coomassie-stained gel pictured in the lower portion of this panel. *B*, the motif PPTPPDF is important for the interaction of GABA_BR1-CT with GABA_A receptors. Additional GABA_BR1-CT fusion proteins were created by adding amino acids between 907 and 934 to the 934–960 truncation in increments of 5–7 residues (1–6). GST pull-down experiments with these truncations revealed that addition of the motif PPTPPDF to the 934–960 truncation restored the association with GABA_A receptors. *C*, the GABA_BR1-CT is not necessary for GABA_BR1 association with GABA_A receptors. Full-length GABA_BR1 (R1) and GABA_BR1 lacking the C terminus (R1TL) were both able to immunoprecipitate (IP) GABA_A receptor subunit immunoreactivity from HEK-293 cells transfected with either full-length HA-GABA_BR1 or the HA-GABA_BR1TL mutant along with α1β2γ2S GABA_A receptors. *D*, the ICL of the γ2S subunit is sufficient to mediate the interaction with GABA_BR1. Control GST as well as the ICL regions of α1, β2, and γ2S subunits fused to GST were incubated with transfected HEK-293 cell lysates and assessed for their ability to pull down GABA_BR1. The γ2S ICL pulled down significant GABA_BR1 immunoreactivity, whereas control GST alone, α1 ICL, and β2 ICL did not detectably pull down GABA_BR1. All data from the panels of this figure are representative of at least three independent experiments.

sion assay. When GABA_BR1 and GABA_BR2 were expressed alone, no change in receptor surface expression was observed following a 30-min stimulation with 100 μM GABA, consistent with previous reports of a lack of agonist-induced GABA_B receptor internalization in HEK-293 cells (42). Strikingly,

however, GABA_BR1/GABA_BR2 receptors exhibited robust internalization (>10%) when co-expressed with the γ2S subunit and stimulated with GABA under identical conditions (Fig. 7E). These data reveal that co-expression with the GABA_A receptor γ2S subunit confers the capacity for agonist-

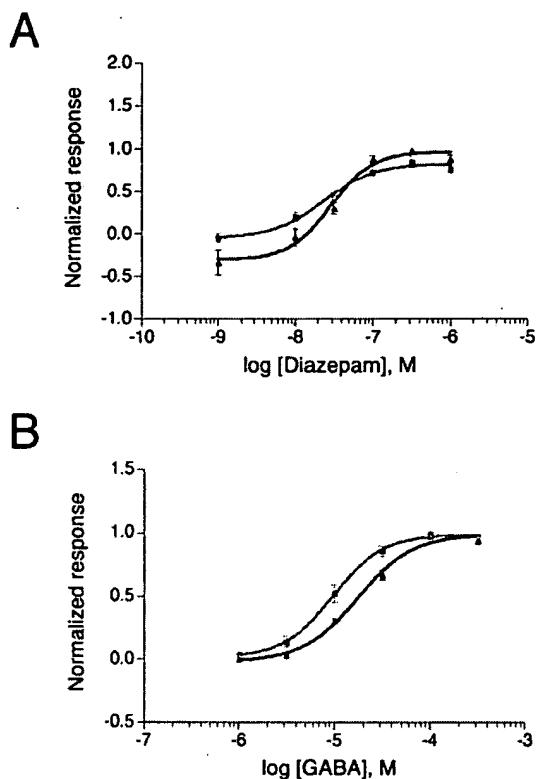


FIG. 6. GABA_BR1 co-expression with GABA_A receptors alters the potency of GABA but not diazepam. *A*, oocytes were injected with cRNAs encoding GABA_A receptor subunits ($\alpha 1\beta 2\gamma 2S$), in the absence (triangles) or presence (squares) of co-injected GABA_BR1 cRNA. The oocytes were stimulated with a single concentration of GABA (10 μ M) and increasing concentrations of diazepam (1, 10, 30, 100, 300, and 1000 nM). The EC₅₀ for diazepam modulation of GABA_A receptor-mediated current in oocytes expressing GABA_A receptors alone was not significantly different from EC₅₀ values derived from oocytes in which GABA_A receptors were co-expressed with GABA_BR1. *B*, oocytes were stimulated with increasing concentrations of GABA (1, 3, 10, 30, 100, and 300 μ M). The EC₅₀ for GABA-induced currents in oocytes expressing GABA_A receptors alone was significantly lower ($p < 0.01$) than EC₅₀ values for GABA-induced currents in oocytes co-expressing GABA_A receptors with GABA_BR1. Data are represented as mean \pm S.E., as determined in 5–10 independent experiments.

promoted internalization to GABA_B receptors expressed in heterologous cells.

DISCUSSION

It has been shown previously that the GPCR GABA_BR1 requires co-expression with another GPCR, GABA_BR2, for efficient trafficking to the plasma membrane (7, 9–11). Here we show that a ligand-gated ion channel subunit, the GABA_A receptor $\gamma 2S$ subunit, can promote the cell surface expression of GABA_BR1 in the absence of GABA_BR2. Although we did not find any evidence that GABA_BR1 and the $\gamma 2S$ subunit can form functional receptors by themselves, we did observe that co-expression of GABA_BR1/GABA_BR2 with the $\gamma 2S$ subunit allows for agonist-promoted internalization of the GABA_B receptors. These findings reveal that the GABA_A receptor $\gamma 2S$ subunit regulates GABA_B receptor trafficking in multiple ways, both promoting GABA_BR1 surface expression in the absence of GABA_BR2 as well as enhancing GABA_BR1 endocytosis in the presence of GABA_BR2.

Because GABA_A and GABA_B receptors are both activated by GABA, our observations that these two receptor types can associate in cells suggests a mechanism whereby neuronal responses to GABA may be coordinated through the formation of complexes containing multiple GABA receptor subtypes.

There have been a handful of previous reports (33–35) describing heterodimerization between GPCRs and ligand-gated ion channels as a mechanism of receptor-receptor cross-talk, but these earlier studies described interactions between receptors activated by distinct neurotransmitters. GABA_A and GABA_B receptors are not only activated by the same transmitter, they are also both found concentrated at symmetric post-synaptic junctions and extensively co-localized in many regions of the mammalian central nervous system (45, 46). In addition to being co-localized at symmetric synapses, GABA_A and GABA_B receptors may also be found together extrasynaptically, as it has been shown that a significant fraction of neuronal GABA_A receptor $\gamma 2$ subunits (47) and GABA_B receptors (45, 48, 49) are expressed in extrasynaptic regions. Our co-immunoprecipitation experiments revealed a robust association between GABA_A receptor $\gamma 2$ subunits and GABA_B receptors derived from native brain tissue, strongly suggesting that this interaction does occur *in vivo*.

There are numerous reports describing examples of cross-talk between GABA_A and GABA_B receptors for which the molecular mechanisms are obscure, notably the ability of GABA_A and GABA_B receptors to mutually influence each other's ligand binding properties (23–25) and signaling activity (24, 26–29). Numerous precedents in the literature illustrate that modulation of signaling, endocytosis, and/or pharmacology can result from direct interactions between receptors (30, 31, 33–35). The physical association between GABA_A and GABA_B receptors that we have observed may play an analogous role in underlying physiological cross-talk between the two receptor types. Along these lines, we found that co-expression with GABA_BR1 modestly increased the potency of GABA acting at GABA_A receptors expressed in oocytes. This change in properties might be due to the physical association between the two receptors directly producing a conformational change in the GABA_A receptor complex that enhances its affinity for GABA, or alternatively might be due to an indirect influence of GABA_BR1 on interactions between the various GABA_A receptor subunits. It is well known that GABA_A receptor binding sites are extremely heterogeneous in brain tissue due to the large amount of GABA_A receptor subunit diversity (2, 50). Our data indicate that GABA_A receptor association with GPCRs such as the GABA_B receptor can subtly modulate GABA_A receptor properties and thereby serve to further increase the functional heterogeneity of GABA_A receptors in the brain. GABA_A receptor functional diversity may also be enhanced via interactions with cytoplasmic proteins such as GABARAP and gephyrin (51, 52), which are known to regulate various aspects of GABA_A receptor clustering, trafficking, and function. Because GABARAP and gephyrin are known to associate with $\gamma 2$ subunits, these proteins may also regulate GABA_A receptor function by influencing receptor interactions with GPCRs such as dopamine receptor 5 (33) and GABA_B receptors.

We found that association with the GABA_A receptor $\gamma 2S$ subunit has profound effects on GABA_BR1 subcellular trafficking in at least two distinct ways. First, we observed that the $\gamma 2S$ subunit promotes GABA_BR1 cell surface expression in the absence of GABA_BR2. Interestingly, the "PPTPPDP" motif on the GABA_BR1-CT that we found to be involved in the GABA_BR1/ $\gamma 2S$ interaction is located in close proximity to the "RSRR" motif that has been identified as a key ER retention signal for GABA_BR1 (7, 8). This ER retention signal may perhaps be masked by interaction with the $\gamma 2S$ subunit in the same manner in which the C-terminal association of GABA_BR2 with GABA_BR1 is believed to mask the signal, thereby promoting the plasma membrane expression of GABA_BR1 (7, 8). However, like the physical interaction between GABA_BR1 and

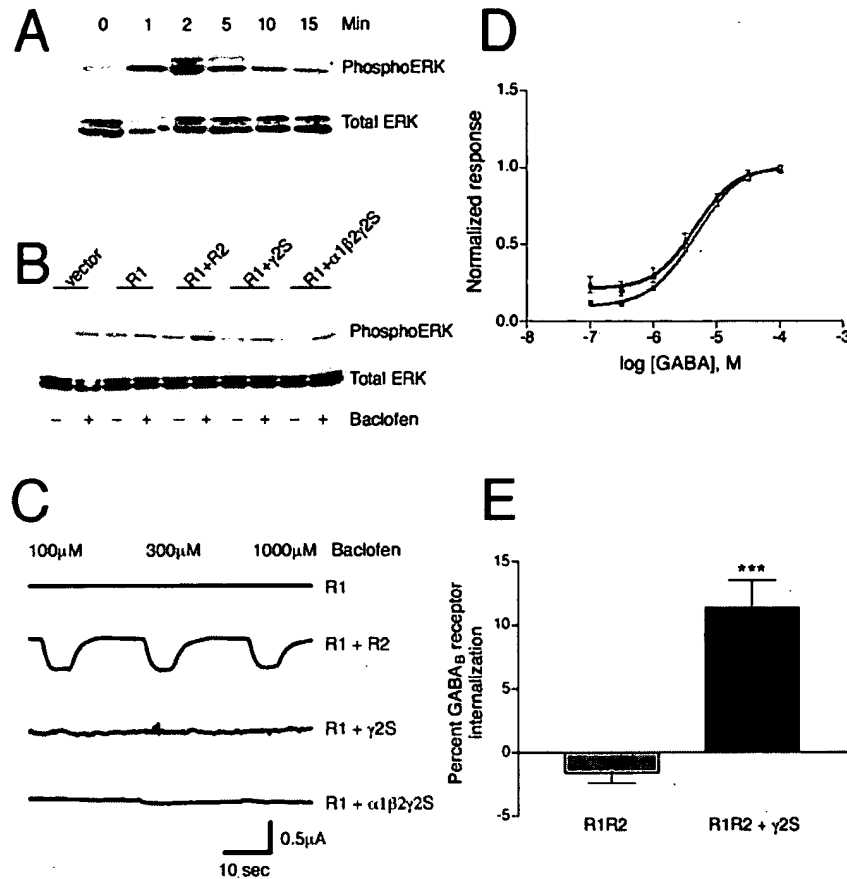


FIG. 7. Association with the GABA_A receptor γ 2S subunit does not confer functionality to GABA_BR1 but does confer agonist-promoted internalization to GABA_BR1/GABA_BR2. *A*, GABA_B receptors can stimulate ERK phosphorylation in HEK-293 cells. GABA_BR1 and GABA_BR2 were transfected into HEK-293 cells and stimulated with 100 μ M baclofen for 1, 2, 5, 10, or 15 min. A robust enhancement of phospho-ERK immunoreactivity was observed between 2 and 5 min as shown in the upper panel. Immunoreactivity for total ERK is shown in the lower panel. *B*, neither GABA_BR1 alone nor GABA_BR1 plus the γ 2S subunit can activate ERK upon agonist stimulation. HEK-293 cells were transfected with empty vector, GABA_BR1 alone, GABA_BR1/GABA_BR2, GABA_BR1/ γ 2S, or GABA_BR1/ α 1 β 2 γ 2S. The various batches of transfected cells were either left untreated or stimulated with baclofen for 5 min. As shown in *A*, the brief stimulation with baclofen induced a significant increase in ERK phosphorylation for cells transfected with GABA_BR1 and GABA_BR2 but did not significantly alter ERK phosphorylation levels for all other cells. These data are representative of three independent experiments. *C*, GABA_BR1 plus the γ 2S subunit cannot activate GIRK currents upon agonist stimulation. Oocytes were injected with GABA_BR1 alone, GABA_BR1/GABA_BR2, GABA_BR1/ γ 2S, or GABA_BR1/ α 1 β 2 γ 2S. GIRK1 and GIRK4 potassium channels were co-injected for all conditions. The GIRK currents were recorded following application of 100 μ M, 300 μ M, and 1 mM baclofen. Large GIRK-mediated currents were observed in oocytes injected with GABA_BR1/GABA_BR2 but were not observed in oocytes injected with GABA_BR1 alone, GABA_BR1/ γ 2S, or GABA_BR1/ α 1 β 2 γ 2S. *D*, co-expression with the γ 2S subunit does not alter the potency of GABA at GABA_BR1/GABA_BR2 receptors. Concentration-response curves for GABA were constructed from oocytes injected with either GABA_BR1/GABA_BR2 (squares) or GABA_BR1/GABA_BR2/ γ 2S (triangles). No significant differences were observed in EC₅₀ values for GABA activation of GABA_BR1/GABA_BR2 versus GABA_BR1/GABA_BR2/ γ 2S. Data are represented as mean \pm S.E. *E*, co-expression with the γ 2S subunit confers agonist-promoted internalization to GABA_B receptors. HEK-293 cells were transfected with GABA_BR1/GABA_BR2 in the presence and absence of the GABA_A receptor γ 2S subunit. The cells were stimulated with 100 μ M GABA for 30 min at room temperature, and the internalization of GABA_B receptors was monitored by tracking the HA-tagged GABA_BR1 subunit with an anti-HA antibody. When examined in the absence of the γ 2S subunit co-expression, the GABA_B receptors did not detectably internalize in response to the GABA treatment. In contrast, the GABA_B receptors co-expressed with the γ 2S subunit exhibited robust internalization in response to the same agonist stimulation. Data are represented as mean \pm S.E. (***) = $p < 0.001$). All data from the panels of this figure are representative of 3–10 independent experiments.

GABA_BR2, the association between GABA_BR1 and the γ 2S subunit is not restricted to the intracellular regions of the two proteins and probably involves the transmembrane domains as well, as indicated by our co-immunoprecipitation experiments where the GABA_BR1 mutant lacking the C terminus was found to associate with GABA_A receptors almost as well as wild-type GABA_BR1. A second effect of the GABA_BR1/ γ 2S interaction is that co-expression with the γ 2S subunit can confer to GABA_BR1/GABA_BR2 receptors the ability to internalize in response to agonist stimulation. Agonist-promoted internalization of GPCRs is known to play a key role in the regulation of receptor desensitization and resensitization (53), but GABA_B receptors expressed in heterologous cells have been found to neither internalize nor desensitize in response to agonist treatment (42). Because GABA_B receptors are known to exhibit robust

desensitization in cerebellar granule cells (42), it seems likely that neurons must express one or more regulatory proteins that are absent from HEK-293 cells and required for agonist-promoted internalization and desensitization of GABA_B receptors. Examples of such regulatory proteins may include G protein-coupled receptor kinase-4 (42) and the GABA_A receptor γ 2S subunit, which is abundantly expressed in cerebellar granule cells (54) and, according to our data, capable of conferring agonist-promoted internalization to heterologously expressed GABA_B receptors.

In summary, our findings reveal that the γ 2S subunit plays a dual role in the trafficking of GABA_B receptors. The γ 2S subunit both supports GABA_BR1 cell surface expression in the absence of GABA_BR2 and also facilitates the removal of GABA_B receptors from the cell surface upon agonist stimulation. Both

of these actions are consistent with observations that the $\gamma 2S$ subunit exhibits constitutive recycling between the plasma membrane and intracellular compartments when expressed alone in heterologous cells (38, 43). In addition to regulating GABA_B receptor trafficking, the interaction between GABA_A and GABA_B receptors reported here also provides a novel mechanism potentially underlying cross-talk and mutual regulation between these two different classes of GABA receptor.

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